

U.S. Application No.: 10/516,361

RESPONSE

Reply to Election/Restrictions Requirement of 01/14/2008

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Attorney Docket No: 3875-033

**REMARKS**

Claims 63-118 are pending in the application.

In response to the election/restrictions requirement on pages 2-3 of the Office action, Applicants elect Group I, claims 63-98, 110-116, and claim 118 in part, drawn to methods of detection of target nucleic acid sequences using a pair of primers separated on two opposite strands by 0-25 nucleotides, for prosecution with traverse because Applicants believe that both Group I and Group II reside within the same inventive concept as will be discussed below in detail.

In response to the restriction subgroups requirement on pages 4-5 of the Office action, Applicants elect SEQ IDs 19, 25; 23, 24 for prosecution with traverse because Applicants believe that a restriction requirement is improper for Markush type claims.

Applicants also reserve the right to file a divisional application for the non-elected claims.

Applicants submit that the present invention does not intend to claim the process of nucleic acid amplification, rather only the use of the method of nucleic acid amplification. Hence, it is Applicants' opinion that the present invention does not overlap with US Patent No. 4,683,195 (Mullis et al., hereinafter "Mullis"). The detection methods described by Mullis and the present invention differ in the basic approach of detection, as well as method of signal generation for detection, though both use the same method of sequence amplification. The present invention cannot overlap with Mullis just because of selection of amplification of segment close to the sizes used in it. Sequence of any length up to 3 KB can be easily amplified, and for probe hybridization based detection any length can be amplified. In Mullis even the smallest sequence that has been subjected to full amplification is outside the size domain used in the present

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invention. In the present invention, amplification primers are selected such that in the fragment amplified by them the 3' ends of the primers are separated by 0–25 base pairs. There is a technical effect of such selection, namely providing solution to the problem of background amplification product. It is not the amplification of a sequence of a length; it is the amplification of a sequence in a specific size domain (primer dimer product size) that brings a special technical effect of a simple and efficient solution to the problems of the prior art (non-specific or background amplification product and primer dimer formation problem) that was neither thought before nor observed or indicated in any of the prior art document including Mullis. It does not require any additional oligonucleotide probe other than two primers. It does not require any separation step. The present invention is a simple, cheaper, efficient, less time consuming (high throughput), single step homogeneous method of detection and quantification of a nucleic acid sequence without use of any separation step or employment of additional probe hybridization. It also allows quantitative detection of a sequence, which is not taught or intended in Mullis. It is an improvement over the method of sequence detection of Mullis.

In the nucleic acid amplification reaction primer dimer and non-specific or background amplification products in addition to the specific amplification product are also formed. In many occasions formation of primer dimer in an amplification reaction also lead to the failure of the amplification reaction. Primer dimer is a nucleic acid amplification product in which 3' ends of the two primers are separated by approximately – 15/- 10 to + 40/+ 35 base pairs. Non-specific amplification products are formed due to non-specific priming of the nucleic acid synthesis. Formation of primer dimer and non-specific amplification product has been a problem in the nucleic acid amplification reaction. Hence as a solution to the above problems, Mullis uses a probe, which hybridizes to the sequence of interest only under appropriate condition. This is an additional step and requires application of an additional labeled oligonucleotide probe. Further, it also require a separation step hence time consuming. Application of high stringency of primer

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annealing has also been suggested in the literature as a solution to the problem of non-specific or background amplification products in the nucleic acid amplification reaction. Neither Mullis nor the high stringency amplification provide any solution to the problem of amplification reaction failure due to primer dimer formation. In addition, the use of probe as well as high stringency of primer annealing either inhibit or make the nucleic acid amplification reaction less efficient reducing sensitivity and at time resulting in failure of the amplification reaction. For the detection of a specific nucleic acid sequence in a sample, amplification of nucleic acids of the sample and application of probe of different types have been used. Since the nucleic acid content in any sample is very little, amplification of the same is essential for its detection, and as nucleic acid amplification reactions have been resulting in amplification of non-specific sequences, the use of probe have also been essential for differentiating specific sequence from non-specific or background amplification sequences. Because of this detection of the presence of a nucleic acid sequence using nucleic acid amplification and probe has been used in the method of Mullis and as well has been improved further by using different types of probe in later documents. Until the present invention, detection of specific nucleic acid sequence using nucleic acid amplification has been dependent on use of probe and application of separation technique. **The present invention in a simple approach just by selecting amplification of an amplification product close to the size of primer dimer solves all the problems of the prior art in a single step.**

The present invention does not reside in the use of additional probe hybridization, or separation and fluorescent staining. To the contrary, the present invention resides in providing a simple specific detection and quantification in homogeneous phase, i.e., without any separation, with high throughput, in a single step without use of any probe and signal is generated mainly through FRET. In this method of sequence detection, the primers are configured in a manner such that in the amplification product 3' ends of them are separated by 0 to 25 base pairs, thus allowing detection as well as quantification of a sequence without the use of a probe or any separation step; i.e., sequence amplification using two primers alone is enough for specific

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sequence detection. The cited art Mullis never teaches separation of the primers in the amplification product by 0 to 25 base pairs or any such selection of primers; it does not teach the detection of specific sequence solely by nucleic acid amplification; it does not teach about the detection of a sequence by nucleic acid amplification without any separation step; it does not teach quantification of the sequence; it does not teach about special effect or the solutions provided by the above selection process and also does not teach about the amplification of a product of the size of primer dimer.

Claim 1 of Mullis, which is specifically cited by the Examiner, teaches the general process of amplification of different segments using primers and additionally hybridization of the probe with the product to detect presence of the sequence of interest, which is different from the method of the present invention and requires additional steps of probe hybridization. The present invention does not require any probe.

Moreover, Figure 4.1 of Mullis, which shows the cycles of denaturation, primer annealing and extension, does not reveal an amplification product where the primers are separated by 0 to 25 base pairs. To the contrary, the separation is 64 (195 - 131) base pairs. It has been clearly mentioned in column 21, lines 24 - 25 of Example 2 that the desired sequence to be amplified was 94 base pair sequence where the primers are of the lengths 15 bases each. This is the minimum size that has been amplified fully from any sample in Mullis.

In example 1, a 25 base pair isolated nucleic acid fragment has been amplified in a limited number of cycles (15) for isotopically labeled molecular weight marker generation using two primers that encompass the total length with separation of one base, using primers of length 11 and 13 bases long. Amplification of a sequence as small as 25 base pair can be relevant for sequence detection by probe hybridization only, but not relevant to sequence detection by amplification alone or amplification and separation as primer as small as 11 base long will

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amplify a lot of non-specific products and such small product can not be distinguished from primer dimer or primers by gel electrophoresis. The primer itself of Mullis contain 15 to 25 nucleotides which may have made the Examiner to think that it teaches or motivates the present invention. To the contrary, in the present invention, the primers are used on the strands in a manner that after amplification of a complex nucleic acid sample they would provide a product where 3' ends of the primers are separated by 0 to 25 base pairs, which make the way for detection of a specific sequence by FRET without use of any probe or separation step; this is neither taught nor motivated by Mullis.

Point is that it is not the amplification of a specific size. It is the detection of a sequence specifically and conveniently. Once the process is known sequence of any length can be amplified. Question is how it is used. In Mullis, the amplified sequence has been used for hybridization to probe for generation of signal in order to detect the specific sequence in presence of all non-specific or background amplification products. It is not a very convenient method and hence has necessitated improvement. In the present invention, amplification of a sequence has been so chosen or configured in such a way that no non-specific or background or primer dimer product is formed. It provides a simple solution to the main problem of the field, i.e., background or non-specific amplification product formation in a nucleic acid amplification reaction, hence there is no need of any probe or any separation step and allows generation of FRET signal, a preferred mode of signal generation. This has been possible due to the selection or design or configuration of the two primers of the amplification reaction. The primers are selected in such a manner that the primers are on two opposite strands and are separated by 0 – 25 base pairs in the amplification product. The product thus formed is close to the size of primer dimer; in a primer dimer 3' ends of the two primers are separated by – 15 / - 10 to + 40 / 35 base pair. In many instances formation of primer dimer leads to the failure of the amplification of a specific sequence. Selection of amplification of a sequence in this size range eliminates formation of primer dimer. It has been mentioned or intended or motivated or indicated anywhere in

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**Mullis to amplify a sequence where the 3' ends of the two primers are separated by 0 – 25 base pair so that the above technical solution of the prior art can be achieved and FRET based signal generation can employed.**

**The smallest segment that has been subjected to full amplification is the 94 base pair segment in which the 3' ends of the two primers are separated by 64 (195 – 131) base pairs as mentioned in Example 2 (col. 21 lines 24 – 25) of Mullis. The smallest fragment that has been amplified is a 25 base pair fragment in a limited number of cycles (15 cycles). The small fragments that have been amplified have been subjected to a limited number of cycles of less than equal to 15 cycles. The non-specific or primer dimer amplification products are formed after 20 cycles. Because of this application of limited cycles, the technical effects mentioned in the present invention cannot be observed or anticipated by the inventors of Mullis. Further, Mullis teaches the detection of a specific sequence by nucleic acid amplification, separation of the amplification products by electrophoresis and fluorescence staining by DNA intercalating dye ethidium bromide. US Patent 5,994,056 has been granted to Roche Molecular System Inc for homogeneous phase detection and quantification of a nucleic sequence by nucleic acid amplification and fluorescent staining using DNA intercalating dye ethidium bromide. In this also nucleic acid sequence fragments are amplified which has been mentioned in Mullis.**

**In summary, the important point of the present invention is not the amplification of a segment of a specific size, but rather the way of detection of the sequence and technical solutions. Mullis teaches mainly detection by probe hybridization, whereas the present invention teaches detection of sequence mainly by FRET involving fluorescent moieties on primers and without use of any probe.**

**Thus the common feature includes FRET distance between two fluorophores is a spectroscopic parameter and is fixed and known from long time. The FRET distance is less**

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than a few nanometer to 100 nm. Beyond 100 nm no FRET is observed. This distance is equivalent to the length of approximately 0-27 base long nucleic acid sequence. Such distance for FRET between the two fluorophores on an amplification product necessitates the separation of the 3' ends of the two primers, through which they are provided, from 0 to maximum 25 base pairs. This use of two primers labeled separately with fluorophores independent of size selection result in the technical effect or solution over prior art that the non-specific, and primer dimer background amplification products are not formed because of internal fluorophore labeling of the primers alone; it is due to the effect of the labels. Similarly, the use of unlabeled primers amplifying a sequence where their 3' ends are separated by 0-25 base pairs in the amplification product because of such selection result in the technical effect and solution to prior art by eliminating formation of non-specific and primer dimer products.

In the present invention it has been demonstrated for the first time that amplification of an amplification product of the size of that of primer dimer can be used for the detection and quantification of a nucleic acid target sequence and also can be used advantageously.

Selections of such product size give certain advantages. It provides a simple and efficient solution to the problems of the prior art including the two main problems i.e., non-specific amplification product formation, primer dimer formation and associated amplification reaction failure resulting in loss of sensitivity, requirement of much optimization of the reaction condition, addition of additives etc. The technical effect of the selection of amplification product of the size of the primer dimer is that:

- (i) Target nucleic acid sequence primer dimer is not formed thus improves the sensitivity by eliminating primer dimer formation and amplification reaction failure..

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- (ii) Non-specific amplification products are not formed even in relaxed stringency of primer annealing, which improves the specificity. The sensitivity is also increased, as the loss of sensitivity due to higher stringency of primer annealing does not arise.
- (iii) There is no need for use of a probe for the detection of the specific sequence, this in turn increases the sensitivity as use of probe slows down the amplification process.
- (iv) It does not require any separation of the amplification product for the detection of a sequence.
- (v) The yield of the amplification is unusually high, ten to thirty times more than that of usual amplification reactions. This improves the sensitivity of detection to a great extent.
- (vi) It is much faster and more efficient.

In this size range amplification of a segment where 3' ends of the two primers are separated by 0-25 base pair in the amplification product has been selected. The above technical effect is most prominent at the above size range.

Because of the above technical effect (non-formation of primer dimer and non-specific amplification product) of selection of the amplification of the size of primer dimer, the amplification/amplification product can be monitored in real time in many different ways, including providing intercalating dyes like ethidium bromide, SYBR green, Fluorophore labeled primer and intercalating dye or fluorophore labeled nucleotide, and using both the forward and the reverse amplification primers labeled with a donor or an acceptor fluorophore, which come within the FRET distance on amplification of the specific amplification product. It also allows monitoring of nucleic acid amplification including PCR, triamplification in real time for absolute quantification.

Use of primers labeled with fluorophore in nucleic acid amplification also give above technical effects except slight reduction of yield due to the labeling of the primers. Hence except

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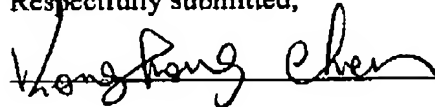
yield of amplification product, the technical effects of primer selection and labeling jointly show enhanced effect.

Mullis neither teaches selection of the amplification of a nucleic acid segment where the 3' end of the primers remain separated by 0-25 base pairs in the amplification product nor teaches the above technical aspects of the present invention.

The common inventive feature of claims of group I and group II is providing a process for detection of target nucleic acid sequence using primers which are present on opposite strands in a manner that in the amplification product they are separated by 0 to 25 nucleotides which is an improvement on the process of Mullis. This feature of manipulating the primers on the strands in a manner such that they would be at specific nucleotides distance in the amplification product is present in claims of both groups which allows better detection of target sequence and is not taught in the cited prior art. Accordingly, both groups I and II reside in the same inventive concept.

Favorable consideration and early issuance of an Office action on the merits are respectfully requested. Should further issues remain prior to allowance, the Examiner is respectfully requested to contact the undersigned at the indicated telephone number.

Respectfully submitted,



Dated: April 14, 2008

Yonghong Chen  
Registration No. 56,150  
AKERMAN SENTERFITT  
222 Lakeview Avenue, Suite 400  
West Palm Beach FL 33401  
Telephone: 561.671.3620

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